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☐ Additional inventors are being named on the _____ separately numbered sheets attached hereto.

TITLE OF THE INVENTION (280 characters max)

UTILIZATION OF STEM CELL AND FIBROBLAST COMBINED PRODUCTS AND NUTRIENTS IN TOPICAL COMPOSITIONS

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ENCLOSED APPLICATION PARTS (check all that apply)

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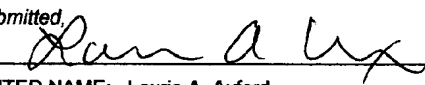
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- ☒ No.
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Respectfully submitted,

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UTILIZATION OF STEM CELL AND FIBROBLAST COMBINED PRODUCTS AND NUTRIENTS IN TOPICAL COMPOSITIONS

by

Jeffrey SEBASTIAN and Quynh SEBASTIAN

FIELD OF THE INVENTION

[0001] The present invention relates generally to cellular products and nutrients, and their use in the field of cosmetics. More specifically, it relates to a formulation containing stem cell and fibroblast combined products and nutrients for use in a topical composition.

BACKGROUND OF THE INVENTION

[0002] Stem cells are the starting point for human and animal development, and are unique in their capacity for self-renewal and potential to develop into specialized cell types.

[0003] Stem cells can be broadly categorized into two types:: pluripotent stem cells, which include embryonic stem cells (ES) and embryonic germ cells (EG), and multipotent precursor cells (MPC). Embryonic stem cells are derived from the inner cell mass (ICM) of the blastocyst, whereas embryonic germ cells originate from primordial germ cells of the gonadal ridge in the 5 to 10 week-old stage where there is a transition from embryo to fetus . Multipotent precursor cells, on the other hand, can be found in embryonic, fetal, and adult tissues, and include the so-called "adult stem cells". Pluripotent stem cells have the ability to proliferate indefinitely *in vitro*, as well as the ability to give rise to different types of cells arising from all three germ layers (ecto-, meso-, endoderm). In contrast, a multipotent precursor cell is capable of differentiation primarily into the specialized tissue from which it originated, and its capacity for self-renewal is typically limited to the lifetime of the organism. Sources of multipotent precursor cells include essentially all tissue types, although they are primarily isolated from bone marrow, blood, liver, skin, intestinal lining, pancreas, brain, skeletal muscle, dental pulp, and adipose tissue. Unlike ES cells, most multipotent precursor cell populations are not clonally derived and consist of a mixture of cell types. It has been reported that it might be possible to revert AS cells back to a state like that of ES cells with the ability to differentiate into different types of cells. Therefore, they may have some "plasticity", in which they have a capacity to form other tissue types, given the proper

environment or stimulation. However, Qui-Long, et al. report that the AS cells take on characteristics of ES cells only after combining with them (Qi-Long, Y., *et al.*, 2002).

[0004] Taking into consideration these differences, most scientists focus on using ES cells in their research. ES cells were first derived from the inner cell mass of mouse blastocyst in the early 1980s (Evans, M. J. and Kauffman, M. H., 1981; Gail, R. M., 1981). More recently, primordial germ cells, cells of the early embryo that eventually differentiate into sperm and oocytes, were found to give rise to cells with characteristics of ES cells and were designated embryonic germ (EG) cells to denote their tissue origin (Shamblott, M. J., 1998). As used herein, the term “stem cell” includes ES cells, EG cells, and multipotent precursor cells, including ‘adult’ stem cells.

[0005] The most useful feature of ES cells is their pluripotency. They retain the ability to differentiate when cultured, and can be induced to differentiate into any of the more than 200 cell types under appropriate culture conditions. Being injected into immunocompromised animals, ES cells form teratomas composed of multiple lineages (Bradley, A., *et al.*, 1984). The combination of such unique properties have made these cells a basic research tool as well as a good source of cell populations for new clinical therapies. They have been used extensively in studies of embryogenesis, identification of gene function and development in the mammal.

[0006] Although the majority of stem cell research has come from animals, the first descriptions of successful isolation and growth of stem cells from early human embryos occurred in 1994 (Hogan, B., 1994) and subsequently in 1998 (Thompson J., 1998). These human ES cells were isolated from fertilized embryos less than a week old. Using 14 blastocytes obtained from donated, surplus embryos produced by *in vitro* fertilization, five independent stem cell lines were developed. These cell lines were capable of being propagated indefinitely *in vitro* as “undifferentiated” ES cells, or under the proper conditions differentiated into specialized cell types from all three germ layers. Additional characteristics of human ES cells include a stable diploid karyotype and the capacity to integrate into all fetal tissues during development.

[0007] Another application of human ES cell technology is in the area of drug discovery. The ability to grow pure populations of specific cell types offers a proving ground for chemical compounds that may have medical importance. Treating specific cell types with chemicals and measuring their response offers a short-cut to identify chemicals that can be used to treat the diseases that involve those specific cell types.

[0008] ES cells are also useful in the study of human development. In particular, the earliest stages of human development are difficult to study. Human ES cells offer insights into developmental events that cannot be studied directly in utero or fully understood through the use of animal models. Understanding the events that occur at the first stages of development has potential clinical significance for preventing or treating birth defects, infertility and pregnancy loss. A thorough knowledge of normal development could ultimately allow the prevention or treatment of abnormal human development. For instance, screening drugs by testing them with cultured human ES cells help reduce the risk of drug-related birth defects.

[0009] In the field of transplantation and cell-based therapy, diseases like juvenile onset diabetes mellitus, Parkinson's disease and some others occur because of defects in one or just a few cells types. Replacing faulty cells with healthy ones offers hope of lifelong or permanent treatment. To date, ES cell-derived cardiomyocytes (Klug, M. G., *et al.* 1998), neural precursors (Brustle, O. *et al.*, 1994) and hematopoietic precursors (Potocnik, A. J., *et al.* 1997) have been transplanted into recipient animals. Recent evidence suggests that conditioned media from embryoid body-derived cells obtained from human embryonic germ cells facilitates recovery from diffuse motor neuron injury, which in part may be due the presence of transforming growth factor-alpha (TGF- α) and brain-derived neurotrophic factor (BDNF) (Kerr, D. A., *et al.*, 2003). Although the long-term outcome of such treatments are not yet fully understood, the findings suggest that the transplanted cells are able to successfully integrate in the host organism, and furthermore, may have the capacity to restore normal host function

[0010] Understandably, to be fully employed by modern medicine, these methods have to overcome a number of obstacles, the most significant of which is donor/recipient compatibility and graft rejection. Possible solutions to this problem include the banking of large numbers of ES cell lines encompassing a significant fraction of the histocompatibility types in the population, and/or the genetic modification of the stem cells to make the graft more acceptable to the recipient. In addition, recent cloning studies (Wilmut, I., *et al.*, 1997; Wakayama, T., *et al.*, 1998; and Kato, Y., *et al.*, 1998) suggest a third solution in which nuclear transfer can be used to produce starting material that allows the isolation of individualized ES cell lines. In this situation, the cells used for transplantation would be genetically identical to those of the patient.

[0011] ES cells are usually cultured in a specific growth medium that requires nutrients and growth factors. Following the addition of cells to a medium, the medium needs to be changed at regular intervals due to the depletion of nutrients and growth factors. In order to obtain an ES cell line, the cells have to be removed and replated under the same culture conditions every 7 to 15 days (“subculturing”). ES cells that have proliferated in cell culture for six or more months without differentiating and appear genetically normal, are considered “pluripotent”, and represent an ES cell line. Undifferentiated stem cell lines under these conditions have been shown to divide for more than two years, which is around 300 population doublings.

[0012] The first mouse ES cell lines were obtained in 1981 by Evans (Evans, M.J. and Kaufman, M.H., 1981) and Martin (Martin, G.R., 1981). The first human ES cell lines were obtained independently by two groups of scientists in 1998. Shambloott, et al., reported on the development of a human EG cell line (Shambloott, M.J., *et al.*, 1998) and Thomson, et al., described the isolation of human cells with the properties of ES cells (Thompson, J.A., et al. 1998). It was stated that the new human ES cell lines possess many of the same features found in the animal ES cell lines, including a series of biochemical and molecular markers on their surfaces and inside the cells, and a stable, normal number of chromosomes. Since then, many ES cell lines have been obtained and described by others.

[0013] Typically, “pluripotent” stem cells are isolated and maintained on mitotically inactive feeder layers of fibroblasts. Fibroblasts are cells of mesodermal origin that make the structural fibers and ground substance of connective tissues. Feeder layers of fibroblasts perform a dual role: 1) they release nutrients into the culture medium; and 2) they provide a structural network and a surface on which ES cells can grow. It has been described that these feeder-dependent culture conditions are useful for the isolation of mouse and human ES cells, and such feeder layers have been proven to be important to maintain the ES cells in an undifferentiated state. The importance of feeder cells suggests that they provide a factor that suppresses the differentiation or promotes the self-renewal of “pluripotent” stem cells. However, only a few of such factors have been fully characterized. One of them is a leukemia inhibitory factor (LIF), which is a member of the family of cytokines related to interleukin-6.

[0014] For murine ES cells, LIF can replace the requirement for feeder cells. Importantly, activation of the signaling component of the LIF receptor, glycoprotein 130 (gp 130), is both necessary and sufficient for inhibiting murine cell differentiation. A crucial downstream effector of gp130 is the signal transducer and activator of transcription-3

(STAT3). Other signaling molecules acting downstream of gp130, such as the mitogen-activated protein kinase, seem to actually inhibit ES cell self-renewal.

[0015] In contrast to murine ES cells, the growth of human ES cells may require feeder cells, but does not seem to require LIF. Presumably, this is because the feeder cells produce multiple growth factors. Therefore, it is possible that some of the same downstream signaling molecules required for mouse ES cell growth, including STAT3, are already activated in human ES cells by other factors. The recent development of feeder-independent culture conditions for human ES cells still necessitates the use of conditioned medium from feeder cells indicating either that these cells require factors produced by feeder cells or that feeder cells remove some inhibitory factor from the culture medium. It has also been shown that the conditioned medium from a specific human tumor line contains a number of unique human stem cell growth factors including a novel ES cell growth factor significantly more potent than LIF (Minger, 2003). However, the precise nature of all of the factors required to grow ES cells and their interplay with one another remains unknown.

[0016] The current methods of propagating human ES cells on mouse fibroblast feeder layers and in serum-containing medium place severe restrictions on the ability to grow these cells to suitable numbers and to the quality standards required for human cell therapy. Thus, many researchers are studying new ways of growing human ES cells without mouse feeder cells and using serum-free medium. This is also important because of the risks associated with the transfer of viruses or other macromolecules from mouse cells to human cells.

[0017] Growth of human ES cells under serum-free conditions has been reported by using a serum replacement (SR) supplemented with basic fibroblast growth factor (bFGF). These better-defined culture conditions support the continuous culture of human ES cells while maintaining the ES cell characteristics. Another approach involves growing human ES cells on a Matrigel matrix (Becton, Dickinson & Co., Bedford, MA) with 100% MEF (mouse embryonic fibroblast)-conditioned medium supplemented with 20% serum replacement and bFGF. However, the use of MEF-conditioned medium may still expose the human ES cells to mouse retroviruses. Thus, the use of an entirely animal-free culture systems for the human ES cells is still the preferred approach.

[0018] Animal-free culture systems were described by Richards, et al., who suggested a culture system based on human feeder layers (Richards, *et al.*, 2002). The authors reported the possibility of growing human ES cells on human embryonic fibroblasts or adult fallopian tube epithelial feeder layers. Cultured with these human feeder layers in

medium supplemented with human serum, human ES cells were found to maintain ES cell features, including pluripotency, key morphological characteristics, and expression of cell-surface markers, for at least 20 passages. They also formed teratomas in severely combined immunodeficient mice.

[0019] Another approach involves the growth of human ES cells on feeder layers derived from foreskin using a serum-free medium (Amit *et al.*, 2003). In this culture condition, human ES cells maintain the same features as reported by Richards, *et al.* (*supra*) after prolonged culture of 70 passages (more than 250 doublings). The major advantage of foreskin feeders is their ability to be continuously cultured for over 42 passages, thus enabling proper analysis for foreign agents, genetic modification such as antibiotic resistance, and reduction of the enormous workload involved in the continuous preparation of new feeder lines.

[0020] As discussed above, culturing ES cells requires the presence of various nutrients and is a complex process. Accordingly, one of the useful by-products from culturing ES cells is the cell growth medium. The used medium is called “conditioned” medium, and in most circumstances is discarded. Although some of the nutrients necessary to support cell growth have been depleted, various other proteins have been added. In particular, the proteins secreted by the cells in culture may include growth factors, cytokines and other growth-stimulatory factors. Thus, conditioned medium is enriched by numerous biologically active cell products.

[0021] It has been previously described that the use of conditioned medium enhances survival of ES cells, presumably through the presence of soluble factors. Included among these is Stem Cell Factor (SCF), which produces biological effects by activating signal transduction pathways, which in turn leads to cell survival, proliferation and finally differentiation. It has also been described that “conditioned” cell culture medium compositions can be used in cosmetics (U.S. Patent No. 6,372,494). However, this patent describes that conditioned medium from any type of cell culture can be used.

[0022] Numerous publications describe the use of fibroblast “conditioned” medium. For example, Kawashima, *et al.* (2002) reported enhancing of viability and proliferation of chick circulating primordial germ cells in suspension culture in the presence of embryonic fibroblast-“conditioned” medium. In addition, scleral fibroblasts of the chick embryo were found to secrete autocrine growth factors (Fujioka, M. *et al.*, 1989). Macromolecular factors produced by serum-free medium conditioned by rat fibroblasts were reported as capable of enhancing the survival of functional islet B cells in tissue culture (Rabinovitch, A., *et al.*,

1979). Human fibroblast “conditioned” medium is described to release insulin-like growth factors (IGFs) and IGF-binding proteins. It has also been shown that a highly supplemented fibroblast “conditioned” medium allowed keratinocytes to be grown and subcultured for up to 19 passages (Hager, B. et al., 1999).

[0023] Accordingly, the combined products produced by stem cells and fibroblasts in culture include many substances that are beneficial to cell growth and survival in general. Thus, these combined products provide an ideal ingredient for cosmetic formulations.

[0024] The skin is composed of the epidermis, dermis and hypodermis or cutis. The most superficial layer, the epidermis, is a continually renewing, stratified, squamous epithelium that keratinizes and gives rise to derivative structures (pilosebaceous apparatuses, nails, and sweat glands) called appendages. It functions as a barrier to environmental stressors. The epidermis is primarily composed of keratinocytes arranged into four layers. Viable cells move outwardly from the inner basal layer to form layers of progressively more differentiated cells. Most of the cells in the epidermis have a limited life cycle. However, adult stem cells comprise a small subpopulation that makes up approximately 10 percent of the basal layer population.

[0025] As described previously, ‘adult’ stem cells are “multipotent”, and have a proliferative reserve that lasts the duration of an individual’s lifetime. They have been identified in the interfollicular epidermis as well as in the hair follicle. A single stem cell may give rise to as many as 4 million keratinocytes. Thus, supplying the skin with factors and products that enhance the viability of stem cells specifically in turn enhances the skin’s biological functions at a cellular level.

[0026] The underlying thicker dermis provides the skin with its structural integrity and texture due to the presence of collagen, elastin, and glycosaminoglycans. Young, firm and healthy skin is abundant in organized bundles of collagen. Elastin, which is present in smaller amounts, accounts for the recoil properties of the skin. Aged skin contains textural abnormalities and exhibits loss of elasticity, which are essentially structural breakdowns of the skin’s integrity. It is this “structural” component of the skin that is benefited by the secreted products and growth-enhancing factors of fibroblasts.

[0027] Aging of the skin occurs via intrinsic and extrinsic mechanisms. Genetic background, ethnic history and chronology are predetermined, and therefore unalterable. However, extrinsic factors, like ultraviolet radiation exposure, smoking, poor diet, stress, and pollution can supercede nature and cause further, premature aging of the skin. Clinically,

this damage can manifest as wrinkling, dryness, roughness, pigmentary alteration and textural abnormalities

[0028] Ultraviolet radiation has deleterious effects primarily on the epidermis and the dermis. A complex cascade of both interconnected and independent events occurs; DNA mutations, immunosuppression, inhibition of normal repair mechanisms, accumulation of reactive oxygen species (ROS), and induction of collagen-degrading enzymes (called matrix metalloproteinases [MMP]) all contribute to the appearance of photoaging. In the epidermis, the resident cells become irregular in size and shape, with decreased cell turnover manifesting as dull or dry skin. Solar lentigines and guttate hypomelanosis result from changes in the melanocyte population. Collagen bundles become shortened, disorganized and degraded, which causes an extensive wrinkling. Accumulation of altered elastin fibers and breakdown in the supporting network lead to loss of elasticity of aged skin.

[0029] At the same time, the average life expectancy has substantially increased in the last decades. As skin is the organ that reflects aging in the most visible way, increased significance has been placed on 'cosmeceuticals' containing substances that are active against skin aging.

[0030] Accordingly, there is a need for skin care products that counteract the effects of factors causing skin aging. The present invention pertains to compositions that provide a dual approach to skin care - products and nutrients of embryonic stem cells enhance the skin's ability to rejuvenate on a cellular level and products and nutrients of fibroblasts provide a more structural approach to skin revitalization.

[0031]

SUMMARY OF THE INVENTION

[0032] The present invention relates generally to cellular products and nutrients and their use in cosmetics. More specifically, it relates to a combined formulation containing both stem cell and fibroblast products and nutrients found in conditioned cell medium for use in a topical composition.

[0033] In one embodiment of the present invention, the conditioned stem cell culture medium contains only the products of stem cells, and the conditioned fibroblast cell culture medium contains only the products of fibroblasts. In other words, the medium is from pure cell cultures containing only stem cells and fibroblasts.

[0034] In another embodiment of the present invention, the conditioned medium may be obtained by way of culturing stem cells on fibroblast feeder layers using animal-free

cultures. The cell growth is possible in monolayers or 3-dimensional cultures. Three-dimensional growth constructs are preferred. The biologically active substances secreted by stem cells and fibroblasts are combined in a formulation adapted for topical application.

[0035] In another embodiment of the present invention, the conditioned medium may be obtained by way of culturing stem cells and fibroblasts separately under suitable conditions followed by the combination of both the stem cell and fibroblast conditioned medium into a formulation adapted for topical application.

[0036] In yet another embodiment of the present invention, a conditioned cell medium from fibroblasts is added to a stem cell culture. The conditioned medium from the stem cell culture then contains all of the products and nutrients from fibroblasts, and can then be added to a formulation adapted for topical application.

[0037] In further embodiment of the present invention, the formulation contains products obtained from genetically modified cells.

[0038] In yet a further embodiment of the present invention, the conditioned medium may be processed in order to remove one or more components from the medium. Such elimination may be achieved by way of filtration (for example, size exclusive filtration) or electrophoresis (for example, gel electrophoresis).

DETAILED DESCRIPTION OF THE INVENTION

[0039] The present invention relates to compositions comprising conditioned cell culture medium from both stem cells and fibroblasts. The cells are preferably human cells to reduce the risk of immune response and exposure to animal viruses.

[0040] The term “conditioned cell culture medium” as used herein refers to a medium that contains the nutritional needs of the cell type being cultured and contains secreted cell products. Examples of cell culture medium include, but are not limited to those described in *Cell & Tissue Culture: Laboratory Procedures*, John Wiley & Sons Ltd., Chichester, England, 1966 and *Methods for Preparation of Media, Supplements and Substrates for Serum-Free animal Cell Culture*, Alan R. Liss, New York, 1984, both of which are incorporated by reference herein in their entirety. These methods are well-known to those skilled in the art. The medium may be supplemented by different ingredients, such as serum,

growth factors, vitamins, interleukins, amino acids, sugars, nucleic acid basics, etc., based on the application.

[0041] There are a variety of substances that are naturally secreted by cells, mainly proteins, such as biologically active growth factors, cytokines, interleukins, etc. Cellular cytokines and growth factors are involved in a number of critical cellular processes including cell proliferation, adhesion, morphologic appearance, differentiation, migration, inflammatory responses, angiogenesis, and cell death. Studies have demonstrated that hypoxic stress and injury to cells induce responses including increased levels of mRNA and proteins corresponding to growth factors such as PDGF (platelet-derived growth factor), GFG (fibroblast growth factor) and IGF (insulin-like growth factor).

[0042] Growth factors, such as transforming growth factor- β (TGF- β) are of particular interest from a dermatological point of view. TGF- β has been shown to have an antiproliferating and differentiating effect on epidermal and haematopoietic cells and also enhances wound healing, embryo- and angiogenesis. Moreover, TGF- β increases the immunological properties of neutrophils, granulocytes and monocytes. The wound-healing process is characterized by migration of monocytes, neutrophils, leukocytes and fibroblasts into the wound area, with release of TGF- β resulting in significantly higher tissue levels. This event, in its turn, enhances cell migration into the wound area until a compensatory down-regulation occurs and wound re-epithelization takes place. TGF- β has also been known to increase the expression of extracellular matrix proteins including collagen and fibronectin. Other growth factors, such as TGF- α and BDNF may also be found in conditioned media and may play a role in the recovery of injured motor neurons.

[0043] PDGF increases the rate of cellularity and granulation in tissue formation. Wounds treated with PDGF have the appearance of an early stage of inflammatory response including an increase in neutrophils and macrophage cell types at the wound site. These wounds also show enhanced fibroblast function. Both PDGF and TGF- β have been shown to increase collagen formation, DNA content, and protein levels in animal studies. A number of other growth factors having the ability to induce angiogenesis and wound healing include VEGF, KGF and basic FGF.

[0044] The cells of the present invention may also be genetically modified. Genetic modification of human ES cells is of significant importance for various applications. It may facilitate genetic selection of pure stem cell population or specific types of differentiated cells. Genetically modified human ES cells can be used to deliver and express foreign DNA

in the course of gene therapy. Genetic alterations of human ES cells such as knockout of MHC locus may be used to generate clones that will not induce an immune response after transplantation. Modification of gene expression by human ES cells may be useful for the study of function of new genes, as well as for the creation of human disease models in vitro.

[0045] Cells may be modified to express a target gene product, which is biologically active, or provides a chosen biological function, or possesses anti-cancer, anti-viral, anti-bacterial and/or anti-microbial activity. These expressed gene products may also serve as reporters of a chosen physiological condition. Thus, it was reported that genetically modified ES cells differentiated under special conditions into midbrain dopamine-neuron-like cells, which is beneficial for treatment of the Parkinson's disease. In order to obtain a population of midbrain stem cells that differentiated into dopamine-producing neurons, ES cells were transformed with a rat gene coding for nuclear receptor related -1 (Nurr1) transcription factor, driven by a Cytomegalovirus promoter to make the gene over-express. The ES cells were then subjected to an elaborate 5-stage culture procedure. The resulting cells expressed many molecular, morphological and functional features specific for midbrain dopamine neurons. Genetically engineered mesenchymal stem cells were shown to control bone regeneration. These cells were genetically modified to possess a tetracycline-regulated expression vector encoding human bone marrow protein-2, an osteogenic growth factor (Moutsatsos, I. K. *et al.* Exogenously regulated stem cell-mediated gene therapy for bone regeneration. *J Mol Ther* 3:449-461, 2001). The cells may be genetically modified to express a fluorescent protein (GFP) which serves as a marker.

[0046] U.S. Patent No. 6,576,464 describes pluripotent stem cells that are engineered to permit the elimination of any undifferentiated cells that might remain after production of a batch of differentiated cells. The stem cells are genetically modified by the proprietary telomerase promoter, which causes expression of the genetic sequence in undifferentiated human ES cells. Because only undifferentiated cells express the genetic sequence, if any undifferentiated cells remain after a batch of cells has been processed, they can be targeted and removed from the cell preparation. The technology may be used to provide an additional level of safety for cell therapies made from human ES cells.

[0047] In the present invention, the cells may be cultured in monolayer or in three-dimensional media. Monolayer cell growth involves an artificial substrate, which is bathed in culture medium. The nature of the artificial substrate may be solid and semisolid. The solid substrates may be plastics, preferably disposable plastics. Semisolid gels are usually prepared from agar and collagen.

[0048] Despite the fact that the majority of vertebrate cell cultures *in vitro* are grown as monolayers and maintain a high rate of cell proliferation, this method of cell growing lacks the characteristics of whole tissue *in vivo*. Therefore, three-dimensional medium substrates are preferred for growing cells to be used to replace tissues. They provide an opportunity to investigate morphological and functional cell interactions during the process of cell growing in culture as well as advanced approaches for targeting numerous human diseases.

[0049] It was shown that three-dimensional cell culture has improved the prospect of treating cancer with gene therapy. Anders, *et al.* studied the cell-surface receptors to which adenoviruses bind and demonstrated that in three-dimensional cultures only malignant cells carried large numbers of the receptors (Anders, M. *et al. Proc. Natl Acad. Sci. USA* 100, 1943-1948 (2003)). Yamada directly compared the growth and development of fibroblasts and collagen-secreting cells in two-dimensional and three-dimensional cultures. In three dimensions, the cells moved and divided more quickly, and assumed the characteristic asymmetric shape that fibroblasts have in living tissues (Yamada, K. M. *et al. Science* 294, 1708-1712 (2001)). This is consistent with the fact that many of the cell types have been reported to penetrate the three-dimensional matrix and establish a "tissue-like" histology.

[0050] The well-known and widely used three-dimensional substrates are collagen gel, gelatin sponge, Gelfoam, cellulose sponge, etc. It has been shown that hepatocytes were maintained in primary culture for 2 weeks on nylon meshes coated with a thin collagen layer (Sirica, *et al.* 1980, *Cancer Res.* 40:3259-3267). Another group of researchers demonstrated that three-dimensional collagen gels have been utilized to culture breast epithelium (Yang, *et al.*, 1981, *Cancer Res.* 41:1021-1027) and sympathetic neurons (Ebendal, 1976, *Exp. Cell Res.* 98:159-169). It was also reported that hemopoietic cells, including primary bone marrow cells, were able to grow in a three-dimensional, tantalum-coated porous biomaterial, Cellfoam (Rosenzweig, M. *et al.*, 1997, *Gene Ther* 4:928-936; Bagley, J. *et al.*, 1999 *Exp Hematol* 27:496-504). Swiss mouse fibroblasts were shown to grow in the three-dimensional collagen-polymerized culture (Pizzo, A. *et al.* 2003 Summer Bioengineering Conference, June 25-29, Florida).

[0051] As previously discussed, the conditioned cell medium is enriched by various biologically active substances. To use these substances, it is sometimes necessary to further process the medium. Depending upon the particular application, it may be desirable to isolate and purify some of the ingredients and/or remove the unwanted ones so that optimal biological activity is maintained. Methods used for isolation and purification of particular products from cell culture medium are commonplace. They include, but are not limited to:

desiccation, evaporation, filtration (for example, size exclusive filtration), electrophoresis (for example, gel electrophoresis), chromatography (for example, gel chromatography ion exchange, affinity chromatography, HPLC purification and hydrophobic interaction chromatography), lyophilization, etc. These methods are described in greater detail in *Cell & Tissue Culture; Laboratory Procedures, supra*. The same methods may be employed to achieve removal of one or more components from the conditioned medium. Isolated and purified products are further used as ingredients in skin care compositions.

[0052] The present invention provides compositions that are adapted for topical administration to the skin. This usually involves combining the cell products and nutrients with some form of carrier. Accordingly, most skin care product contain basic elements, optimized galenics, and active substances.

[0053] Basic elements are water, fats and oil, which are the central raw materials for high-quality bases of dermatocosmetic preparations. Fats and oils are of vegetable origin. An example might be coco-mono-glycerid sulphate that is synthesized from coconut oil and glycerine (Behler, A. *et al.*, 1999) and has the properties of an anionic surfactant. An additional group of partial synthetically modified vegetable raw materials are the protein-fatty acid condensates that equally belong to the anionic surfactants and are very well skin-tolerable. The non-ionic alkyl polyglycosides (Hill, K. and Rhode, O., 1999) composed of sugar and fatty alcohol distinguish themselves as well through excellent skin tolerability and a very low irritative effectiveness.

[0054] The idea of optimised galenics is to establish qualified systems for a controlled release of active substances and optimization of their availability in certain skin layers. Examples of such systems are nanodisperse systems as liposomes, nanoemulsions and lipidnanoparticles. It was shown that application of uncharged liposomes causes significant increase in skin hydration (Garei, B.J. *et al.*, 1995). Moreover, liposomes allow a fixing of active substances in the upper skin layers. A washing out and continuing penetration are hereby impeded.

[0055] Nanoemulsions enhance the penetration of cosmetic active substances and intensify their concentration in skin (Garei, B.J. *et al.*, 1995). They improve the barrier function by infiltrating of qualified lipids. Lipidnanoparticles seem to allow an improved stability of chemically unstable active substances as well as their controlled release, enhanced hydration and a good control effect by film formation.

[0056] Optimized galenics may be stabilized by gels or derivatives thereof. Gels are classified as hydro-gels, hydro-dispersion gels with creamy appearance and water-free oleo

gels. Hydro-gels are non-disperse systems containing a liquid component. Hydro-dispersion gels consist of hydrophylic and lipophile disperse phase. From physico-chemical point of view, these gels are disperse systems consisting of at least two components - a solid component and a liquid. The solid component forms a coherent, three-dimensional structure, whereas the liquid is present in an immobilized form as coherent medium in the matrix. Using a hypromellolis instead of usual gel forming polymers allows production of hydro-dispersion gels with particles sizes in sub-micron area. Nanoemulsions stabilized with hypromellosis can be sprayed without difficulties and sterilized without quality loss.

[0057] Oleo gels consist of a lipophile liquid phase. Addition of special copolymer as gel former allows to form highly transparent gels that are qualified as basis for a large range of cosmetic formulations. As the viscosity of these formulations decreases in sphere of the skin temperature, an even spreading of the products on skin is enhanced. Oleo gels have high substantivity with long lasting reduction of trans-epidermal water loss. They also have good stabilizing properties for suspensions and prevent sedimentation of solids, such as zinc oxides even at high temperatures.

[0058] Many dermocosmetics may also contain two or more active substances that are not miscible with each other and only reach a sufficient stability by certain additives. Often such formulations are stabilized with classical emulsifiers of the type of ionic or non-ionic (or zwitterionic) surfactants.

[0059] Active substances of dermocosmetics include a variety of chemical compounds, and can also be added to the formulations of the present invention. On this list are: vitamin A and its derivatives, antioxidant vitamins C and E, coenzyme Q10 and flavonoids, ectoines, DNA-repair enzymes, phytoestrogens, dimethylaminoethanol (DMAE), alpha-lipoic acid, pentapeptides (Pal-KTTPS), matrix metalloproteinase inhibitors (MMPI) and other naturally occurring and synthetic moieties.

[0060] Vitamin A and its derivatives are well known ingredients in moisturizers and anti-aging substances. All retinol derivatives exercise their specific effect on the nuclear receptors and have a complex impact on the skin tissue via their effects on gene expression. This results in synthesis of new collagen by impeding collagenase activity and reorganization of damaged collagen and elastin fibers (Griffith, C., *et al.*, 1993).

[0061] The main use of antioxidant vitamins C (ascorbic acid) and E (α -tocopherol) is to protect skin from oxidative damage caused by reactive oxygen species (ROS). Ascorbic acid is also involved in proline hydroxylation in the area of the protein cord of the collagen molecule. This results in a stimulation of the connecting tissue metabolism with increased

mRNA expression of collagen-synthesizing enzymes and in regeneration of age-related connecting tissue damages. α -Tocopherol belongs to the lipophile anti-oxidants that is able to catch free radicals at cell membranes. Application of vitamin E causes reduction of UVA-induced oxidative stress, significant decrease of peroxidized phospholipids (Lopez-Torres, M. *et al.* 1998), and impeding the gene expression of collagenase by inhibition of proteinase C activity (Ricciarelli, R. *et al.*, 1999).

[0062] Coenzyme Q 10 also reduces the oxidative stress by decreasing collagenase's activity (Hoppe, U., *et al.*, 1999). Flavonoids are polyphenolic compounds found in plants. Important examples are polyphenols with epicatechine, epigallocatechin and epicatechin-3-gallate having photo-protective effect.

[0063] Ectoines comprise a group of chemically differing substances naturally found in bacteria and corresponding to tetrahydropyrimidin-carbonic acid compounds. Ectoines are amphoteric water-binding substances protecting skin from UV radiation. They stabilize proteins, nucleic acids and membranes and also have a hydrating effect *in vivo* (Bunger, J. *et al.*, 2000).

[0064] DNA-repair enzymes presented by photolyases, T4N5-endonucleases and topoisomerases. These enzymes work in combination with liposomal emulsions and reduce quantity of UV-induced pyrimidine dimers.

[0065] Phytoestrogens presented by different substances of vegetable origin, such as isoflavone, coumestane and lignane, as, for example, in soy and soy products, green tea and ginseng (Whitten, P.L. and Patisaul, H.B., 2001). These compounds have positive effect on skin elasticity and wrinkle formation, although the mechanism of their action is still unknown.

[0066] Methods of manufacturing cosmetic products are well known. The forms of cosmetic products include, but are not limited, to solid, lyophilisate, powder, gel, or film forms. Each formulation type offers benefits relating to their physico-chemical and skin physiological properties. They are further presented by creams, compacts, lotions, sprays, emulsions, cleansers, etc.

[0067] An exemplary solid cosmetic product is zinc oxide and titanium dioxide used as dispersed particles in sunscreens. U.S. Patent No. 6,106,855 discloses oil-in-water emulsions stabilized by the presence of insoluble protein in the aqueous phase. By varying the amount of insoluble protein the emulsions may be made liquid, semisolid or solid. The disclosed emulsions may be medicated with hydrophilic or hydrophobic pharmacologically active agents and are useful as wound dressings or ointments. Water-soluble quaternary

ammonium cellulose derivatives of controlled charge density are shown to be useful in cosmetic preparations, such as hair and skin formulations. They are used as thickeners, conditioners, film formers, fixatives, emulsifiers, or additives in hair or skin formulations to improve combing, manageability, body, curl retention, moisture resistance, and binding of ingredients to keratin. Hydrophilic polymeric hydro-gels are used as film formers for cosmetic topical applications.

[0068] Regardless their physico-chemical properties, all cosmetic products must follow the same principles of safety as well as satisfy the requirements of the effectiveness of cosmetics. Proven claims are smoothening and tightening, moisture enrichment, wrinkle reduction, blood circulation and stimulation of skin, sweat reduction, sun protection, etc.

[0069] A great number of compounds used in cosmetic applications possess not one but a few relevant properties. Thus, lactic acid and lactates are well-known skin moisturizers, rejuvenators and pH regulators. Recently, they were shown to express exfoliating, antimicrobial and skin-lightening properties. Being efficient by themselves, these substances also demonstrate synergistic effect with other active ingredients. Thus, lactic acid sufficiently decreases the irritative effect of salicylic acid. Another example is silicone compounds that are widely used in cosmetic preparations. Based on their viscosity, which can be precisely controlled, the lower viscosity silicone compounds are useful for aerosol applications. The middle viscosity products are traditional silicone oils used as additives in skin care formulations and the higher viscosities are used in barrier products (for example, barrier creams) and in hair serums. Natural lipids (for example, extracted from shea butter and hemp seeds) were proven to provide softness, smoothness and flexibility of the skin; be solvents and vehicles for active ingredients and also pigment grinding bases for decorative cosmetics; and to enhance hair gloss.

[0070] Pantothenic acid (vitamin B5) was shown to statistically enhance epidermal regeneration. According to dermatopharmacokinetic studies, pantothenic acid is able to penetrate the skin after topical application that makes it an ideal candidate for use in the field of wound healing and minimizing the process of scarring, which is beneficial for cosmetic surgery needs.

[0071] Different proteins with enzymatic activity have also been shown to be useful in cosmetic topical applications. One of them is collagenase, a unique proteolytical enzyme, which can selectively hydrolyse insoluble collagen albumen. Thus, collagenase participates in transformation of the connective tissue in the process of its growth and morphogenesis.

[0072] It is also commonplace to include ingredients with anti-acne activity in cosmetics. The formulations can be applied topically, such as gels, creams, lotions, milk and solid preparation. The formulations can be prepared according to conventional methods well known in the art and as further described by "Remington's Pharmaceutical Handbook," Mack Publishing Co., N.Y., USA. Additionally, the formulations may include suitable excipients, such as antioxidants.

[0073]

CLAIMS

We claim:

1. A composition for use as a topical skin care product comprising:
 - a) a conditioned stem cell culture medium;
 - b) a conditioned fibroblast cell culture medium; and
 - c) a delivery vehicle adapted for topical administration.
2. The composition of claim 1, wherein the stem cells are embryonic stem cells.
3. The composition of claim 1, wherein the conditioned stem cell culture medium contains only products from stem cells and wherein the conditioned fibroblast cell culture medium contains only products from fibroblasts cells.
4. The composition of claim 1 produced by a process of culturing stem cells on a fibroblast feeder layer.
5. The composition of claim 1 in a form selected from the group consisting of a solid, a lyophilizate, a powder, a gel, and a film.

6. The composition of claim 1 produced by a process of culturing stem cells and fibroblast cells in a three-dimensional cell culture.
7. The composition of claim 1 produced by a process comprising the steps of:
 - (a) culturing stem cells and fibroblasts separately;
 - (b) removing the conditioned cell culture medium from each; and
 - (c) combining the conditioned cell culture medium from (b) together with a delivery vehicle adapted for topical administration.
8. The composition of claim 1 produced by a process comprising the steps of:
 - (a) culturing stem cells in a conditioned fibroblasts cell medium to form a conditioned stem cell culture medium comprising products of both stem cells and fibroblasts;
 - (b) removing the conditioned stem cell culture medium; and
 - (c) combining the conditioned cell culture medium from (b) together with a delivery vehicle adapted for topical administration.
9. The composition of claim 1, wherein said cultured fibroblast cells are human fibroblast cells.
10. The composition of claim 1, wherein said cultured stem cells are human embryonic stem cells.
11. The composition of claim 1, wherein said cultured stem cells and said cultured fibroblast cells are genetically modified.

ABSTRACT

[0074] The present invention relates generally to cellular products and nutrients, and their use in the field of cosmetics. More specifically, it relates to a formulation containing stem cell and fibroblast combined products and nutrients for use in a topical composition. Such compositions generally also include a delivery vehicle adapted for topical administration, and may include a variety of other ingredients commonly found in skin care products. The products may be in the form of powders, creams, jels, etc.



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